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Characteristics of the Early Phase of Chronicity in Acute Hepatitis B Infection

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The mechanism of development of chronicity after acute hepatitis B infection has not been elucidated fully. Following a single source outbreak of hepatitis B among 79 adult women, three patients (4%) became chronic carriers of hepatitis B virus (HBV). We compared features of the virus and antibody response of the latter three patients with those of 12 HBeAg-positive cases with resolving infection. The virus genotype was D, antigenic subtype ayw_2. Base sequence analysis of S- and C-gene regions revealed no differences between the two groups. During the acute illness the three patients who developed chronicity had a remarkable transient reduction of HBsAg, HBeAg, and HBV DNA levels at 14–20 weeks after infection, the time of HBeAg seroconversion in the patients who cleared the infection. One HBeAg-specific monoclonal antibody (HBOT.95A) used as solid-phase antibody in a sandwich enzyme immunoassay detected an increased HBeAg signal in 2 of the 3 patients that developed chronicity and in 1 of the 12 patients who recovered. The latter patient had an exceptional long period of HBsAg antigenemia. Standard HBeAg assays detected HBeAg in all cases. HBeAg and anti-HBe–positive serum samples from the patients who recovered could inhibit the HBOT.95A response. The results suggest that chronic hepatitis B develops after an interruption of immune clearance. Differentiation of the antibody response to HBeAg may help to find patients with an increased risk for this interrupted immune clearance who might be candidates for an early intervention therapy. ***J. Med. Virol. 57:331–336, 1999.***

KEY WORDS: hepatitis B; acute; chronic; monoclonal; HBeAg; prognosis

INTRODUCTION

It is estimated that hepatitis B virus is responsible for about 300,000 annual infections in the United States, with a presumed risk of chronicity that differs with age, being 90% in newborns, 25% in preschool children, and below 5% in adults [Kane et al., 1989]. Possible mechanisms of developing the carrier state are well described [Barnaba and Balsano, 1992] and vary from host factors such as immunological tolerance, immunesuppression, and major histocompatibility complex (MHC) defects to viral factors such as aberrant viral protein expression, viral antigen processing, and viral mutation.

Since in adults more than 90% of the acute infections are self-limiting, no therapy is given at present to prevent the chronic carrier state. HBsAg screening, hygienic precautions, and vaccination can prevent most of the acute hepatitis B infections. However, unexpected hepatitis B epidemics occur as a result of medical procedures such as vaccination, acupuncture, surgery, bone marrow transplantation, or administration of red blood cells. During a hepatitis B epidemic in a setting of an in vitro fertilization (IVF) procedure, we were able to scrutinize the development of three chronic cases in a cohort of 79 infected patients. The present study is intended to find early viral parameters in order to discriminate between resolving and early chronic hepatitis B and for consideration of early therapeutic intervention.

MATERIALS AND METHODS

Patients

At the end of 1987, 79 patients became infected with hepatitis B virus (HBV) during an in vitro fertilization

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procedure due to a contaminated pool of human serum used in embryo culture; 3 of these patients developed chronic hepatitis B infection [Van Os et al., 1991]. Apart from these three patients, we studied all patients (n = 12) with at least two consecutive HBsAg-, anti-HBc-, and HBeAg-positive samples at first screening in March 1988. Four of the 15 women became pregnant as a result of the IVF treatment. To follow up this small epidemic, blood sampling was performed every 2 weeks for 2 months and monthly until resolution (anti-HBc$^+$, anti-HBe$^+$, anti-HBs$^+$) or in the chronic cases until at least 1 year after infection.

Routine HBV Assays

HBsAg, HBeAg, anti-HBc, anti-HBe, and anti-HBs were determined by microparticle enzyme immunoassay (IMx, Abbott Laboratories, Chicago, IL) and quantified by the use of appropriate standards (Paul Ehrlich Institute standards for HBsAg and HBeAg; WHO Reference preparation for anti-HBs). IgM–anti-HBc was assayed with Corzyme-M (Abbott Laboratories). HBV DNA levels were measured by solution hybridization (Abbott Laboratories). Alanine aminotransferase (ALT) levels were assayed on stored serum samples.

HBeAg Monoclonal Antibody Assays

HBeAg standard assay. This enzyme immunoassay is based on two monoclonal antibodies. Murine monoclonal anti-HBe, MAB 364C8 (Sorin Biomedica, Italy), is coated on the solid phase (microtiter plate). Human monoclonal HBOT.Hu03-horse radish peroxidase (HRPO) conjugate (Organon Teknika, Boxtel, the Netherlands) is used for detection. The test procedure is as follows: 100-μl sample is incubated for 1 hr at 37°C followed by four wash steps using PBS/Tween (pH 7.2). After washing 100-μl conjugate is added in the coated test wells, incubated (1 hr 37°C) and removed with washings as described. Hereafter, 100-μl tetramethyl benzidine (TMB) ready-for-use substrate (Organon Teknika Uniform II substrate) is added to the wells and incubated for 30 min at room temperature. The reaction is stopped by adding 100 μl of 1-M sulfuric acid. Absorbance is measured at 450 nm.

HBeAg selection assay. This enzyme immunoassay is also based on two monoclonal antibodies. Murine monoclonal anti-HBe antibody MAB HBOT.95A (Organon Teknika) is coated to the solid phase. As for the standard assay, HBOT.Hu03-HRPO is used as the conjugate. The test procedure is similar to the standard assay.

Standard assay vs. selection assay. To compare the results of a serum sample in the standard assay and the selection assay, the HBeAg titer at OD 1.0 was read from a dilution curve in the standard assay. In a similar dilution curve of the sample in the selection assay, the optical density was read at a 30-fold higher concentration. This optical density result is denoted as the normalized HBeAg signal in the selection assay.

Inhibition assay. To investigate whether host-derived factors may play a role in masking the HBOT.95A epitope in serum tested negative in the HBe selection assay and positive in another HBeAg assay, inhibition experiments were carried out. Serum obtained during the acute phase of the infection of two chronic cases served as positive control (PC). These sera were diluted 2/3 times using normal human serum. Subsequently, different sera, whether HBeAg-positive or -negative by standard HBeAg assay, and the PC serum were mixed in equal parts, incubated for 8 hr at room temperature and measured in the HBe standard and the HBe selection assay.

Sequence Analysis

DNA was isolated according to Boom et al. [1990]. For S-gene PCR, the following primers were used: nucleotide 56–85, sense: 5′-CCTGCTGGTGGCTCCAGTCCCGGAACAGTA-3′; nucleotide 806–786, antisense: 5′-TTGGTAACAGCGGTATAAAGG-3′. These primers select the genomic region of HBsAg defining the different subtypes of the virus.

For the HBeAg gene, primers covering nucleotides 1778–2430 were used for amplification (nucleotide 1778–1794, sense: 5′CGACGTTGTAAAACGGCCAGTAGGAGGCTGTAGGCATAAAT-3′; nucleotide 2410–2430, antisense: 5′-CAGGAAACAGCTATGACCTTCTGCGACGCGGCGATTGA-3′). The primers were elongated with M13 forward and M13 reverse sequences. PCR products were purified using the Qiaquick or Qiaex protocols (Qiagen, Germany) and analyzed on an Applied 373 automated sequencer (Perkin-Elmer, Norwalk, CT) or an ALF sequencer (Pharmacia Biotechnologies, Uppsala, Sweden).

RESULTS

HBV Subtyping

Subtyping of the HBsAg was performed by sequencing nucleotides corresponding to amino acids 103–196 of the HBsAg small S-protein. In all cases, including the three chronic patients, the HBV genotype was type D [Norder et al., 1994]. This corresponds to the antigenic subtype ayw$_2$ as determined before [Quint et al., 1990].

Acute Hepatitis B

The HBV epidemic was discovered about 4 months after a 6-week use of contaminated serum. Therefore, all stages of infection were seen at first screening of the women at risk. The number of patients with 2–4 consecutive HBeAg-positive blood samples was limited to 15 of the 79 infected cases.

For analysis of the acute phase of the disease in the three cases who developed chronic hepatitis B and the acute cases with recovery, we compared HBsAg, HBeAg, and HBV DNA levels in the sample with the highest level of HBeAg (Table I, patients 1–7) or, if applicable, the first screening sample (patients 8–15). All three parameters varied widely. In the three chronic cases, HBsAg, HBeAg, and HBV DNA levels were similar to that in the recovered cases. However, since we observed a decline in these HBV markers be-

TABLE I. Comparison of Markers of Acute Hepatitis B Infection in Patients Who Resolved Infection and Patients Who Became Chronic Carriers[a]

Patient code	Pregnancy	Sampling week	HBsAg, μg/ml	HBeAg, PEIU/ml	HBV DNA, pg/ml	IgM–anti-HBc, weeks
Recovered cases						
1	No	15	4	831	13	
2	Yes	15	36	840	158	18–22
3	Yes	14	18	1173	171	22–22
4	No	16	1	393	14	16–22
5	No	17	84	2772	113	16–24
6	No	16	27	1915	282	19–19
7	No	15	13	3045	32	15–17
8	No	15	12	2329	354	17–21
9	Yes	14	27	204	217	17–19
10	No	14	3	819	24	17–21
11	No	15	42	693	97	16–25
12	No	13	90	2710	166	15–18
Chronic cases						
13	No	16	16	473	14	16–16
14	Yes	14	42	1873	454	14–25
15	No	15	10	1209	172	

[a]Results are depicted from samples with the highest HBeAg levels whether during follow-up (patients 1–7) or at start of sampling (patients 8–15).

tween week 14 and 20, it is obvious that the peak levels of HBsAg, HBeAg, and HBV DNA of the early acute phase in the chronic cases were missed.

As a marker of acute hepatitis, IgM–anti-HBc was detected in 11 of the 12 acute cases and in 2 of the 3 chronic cases. The period of IgM–anti-HBc positivity varied from <2 to 12 weeks (Table I). For the recovered cases, the median time of HBeAg conversion was week 17 after infection (range, 15–29 weeks); the median time of HBsAg conversion was week 27 after infection (range, 18–44 weeks).

One patient (patient 3, Table I) had a long period of HBeAg antigenemia (until week 29). This patient became pregnant after IVF treatment; HBsAg, HBeAg, and HBV DNA disappeared shortly after delivery. As from week 18, the HBs antigenemia in this patient was characterized by a continuous high level of HBeAg and low level of HBV DNA. Another patient (patient 9) had an extremely long period of HBsAg antigenemia (until week 44) after HBeAg seroconversion at week 18. After a second IVF treatment, this patient gave birth to a twin at week 49 after infection.

The three chronic patients showed minimal levels of HBsAg, HBeAg, and HBV DNA between weeks 15 and 22 after IVF treatment, followed by an increase 2–4 weeks later (Fig. 1). The phenomenon of a transient reduction in HBeAg was also observed in the recovered case with the extended HBeAg antigenemia (Fig. 2, patient 3, filled bars).

HBeAg Heterogeneity

Figure 2 illustrates for the standard enzyme immunoassay the HBeAg level changes in the early acute samples after first screening. In seven cases (patients 1–7), samples were obtained before the maximum level of HBeAg was reached.

In 34 of the early acute samples from the recovered group of patients, sufficient HBeAg reactivity was present as detected by the standard assay to test their reactivity in the selection assay. In the depicted cases, dilutions vary from 1/10 to 1/100 in the selection assay with a fixed dilution per patient; in the standard assay, 10-fold higher dilutions were used systematically. As shown in Figure 2 and confirmed by results in complete dilution series, the HBeAg reactivity in the selection assay (open bars) generally does not follow the changes in the standard assay (filled bars). Elevated levels (>500) of HBeAg in the selection assay were observed in one acute case (patient 9, late HBsAg seroconversion case) and for two of the three chronic cases (patients 13 and 15). Patient 15 had an increased signal only in the first two screening samples, whereas patient 13 demonstrated reactivity for HBOT.95A in six samples over a period of 10 months.

Inhibition of Selective HBeAg Reactivity

The HBeAg-positive samples used in the inhibition experiments were appropriately diluted to fit into the linear range of the assay; samples for inhibition were used undiluted. Results as presented in Table II show that anti-HBe–positive as well as HBeAg-positive serum from the acute cases with recovery was able to inhibit almost completely the HBeAg signal of patients 13 and 15 in the selection assay. A late HBeAg-positive serum (3 years after start of infection) of patient 15 could inhibit 82% of the HBeAg signal of her early serum sample (week 32) and 44% of the signal of patient 13.

Base Sequence Analysis of the HBsAg- and HBe-Antigenic Regions

We studied serum samples from the chronic cases and recovered patients; all of them had the same base sequence in the HBeAg1 determining C-region (amino acids 76–89) [Salfeld et al., 1989] in the acute phase of the disease. Sequence analysis of the chronic cases was

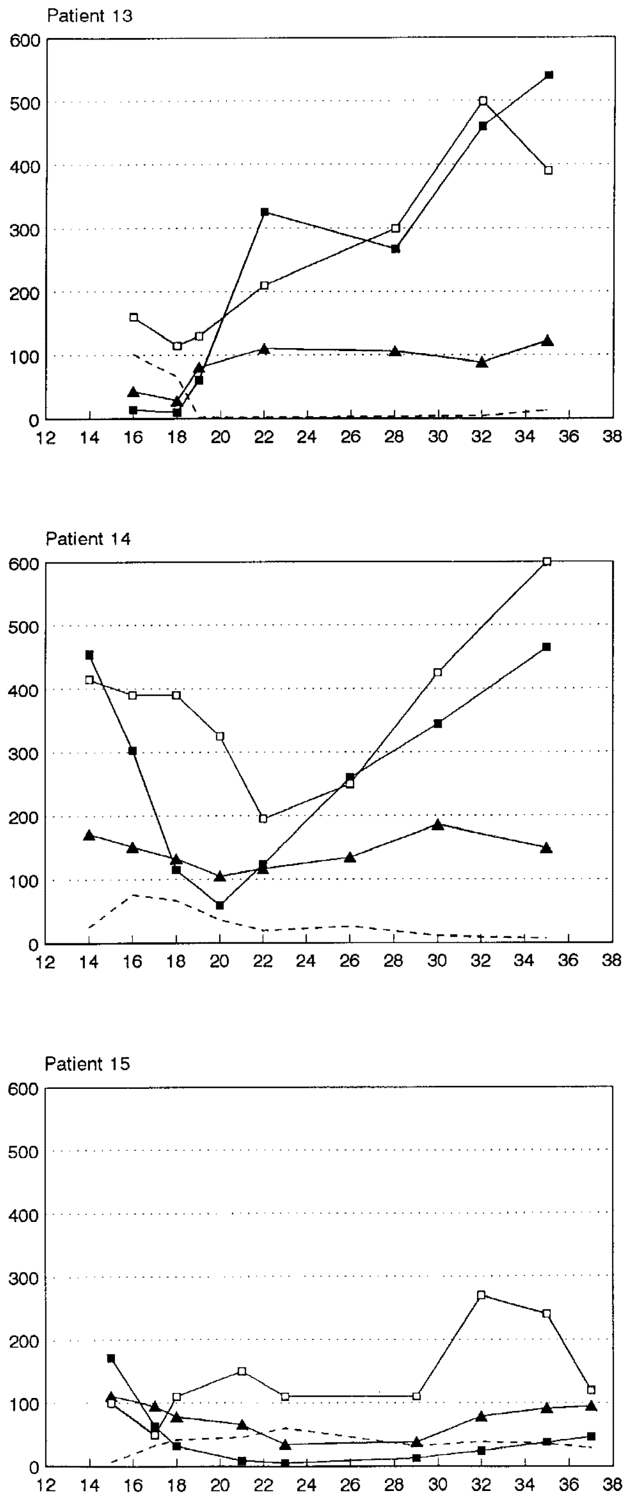

Fig. 1. Quantitation of HBsAg, HBeAg, and HBV DNA in the course of acute infection of the three chronic patients. HBsAg × 10 µg/ml (□); HBeAg × 0.1 PEI U/ml (▲); HBV DNA × 1 pg/ml (■). Dashed line represents ALT level changes. X-axis: time in weeks; Y-axis: arbitrary units.

repeated during the chronic phase of the disease (>6 months after first screening). In all three cases, the HBeAg as well as the HBsAg sequences as determined in the acute phase of the disease were not changed during the course of infection.

Long-Term Follow-Up

Two of the three chronic patients (patients 13 and 14) showed HBeAg seroconversion during or shortly after the first course of interferon, which started about 1 year after infection. The third patient (patient 15) is still HBeAg-positive after several courses with interferon.

DISCUSSION

Studies on the dynamics of hepatitis B virus in acute hepatitis are scarce, in particular regarding the difference between patients with resolution and those that develop chronic hepatitis [Lok et al., 1985; Fong et al., 1989, 1994]. We were able to follow 3 patients who developed chronic disease among a cohort of 79 women with acute hepatitis after infection during IVF treatment.

According to Fong et al. [1994], factors determining the development of chronicity operate during the first weeks of infection. On the other hand, the decline of HBV proteins in the blood of our three cases that developed chronic disease 14–20 weeks after infection suggests an active process of clearance. Probably, this clearance could not be completed due to a suboptimal immune response. Theoretically, this suboptimal immune response may be inherited. Late cases of HBeAg and HBsAg seroconversion in acute and chronic hepatitis, spontaneous or therapy-induced, suggest that the weakness of the immune response may be temporary. Two of the chronic cases had an HBeAg seroconversion during or just after one course of interferon therapy. Some cases of temporary weakness may be related to pregnancy that is known to be a state of limited immune reactivity. Indeed, from our four cases with pregnancy (Table I), two patients showed late HBeAg or HBsAg seroconversion and one patient developed chronicity.

Another point of interest is the finding of an HBeAg-specific epitope that behaved differently in our experimental HBeAg assay (selection assay) compared to the standard commercial HBeAg assays.

Elevated levels of HBeAg were observed in the experimental assay in one case of acute hepatitis with delayed recovery and two of the three patients that became chronic. The enhanced signals in the experimental assay of the two cases that developed chronicity could be inhibited by HBeAg-positive serum or anti-HBe–positive serum lacking the HBOT.95a-specific signal.

Based on these observations, we suggest that elevated signals in the selection assay result from a binding of serum HBeAg to solid-phase monoclonal anti-HBe (HBOT.95A) with epitopes that are in the case of the early HBeAg-positive phase of acute resolving

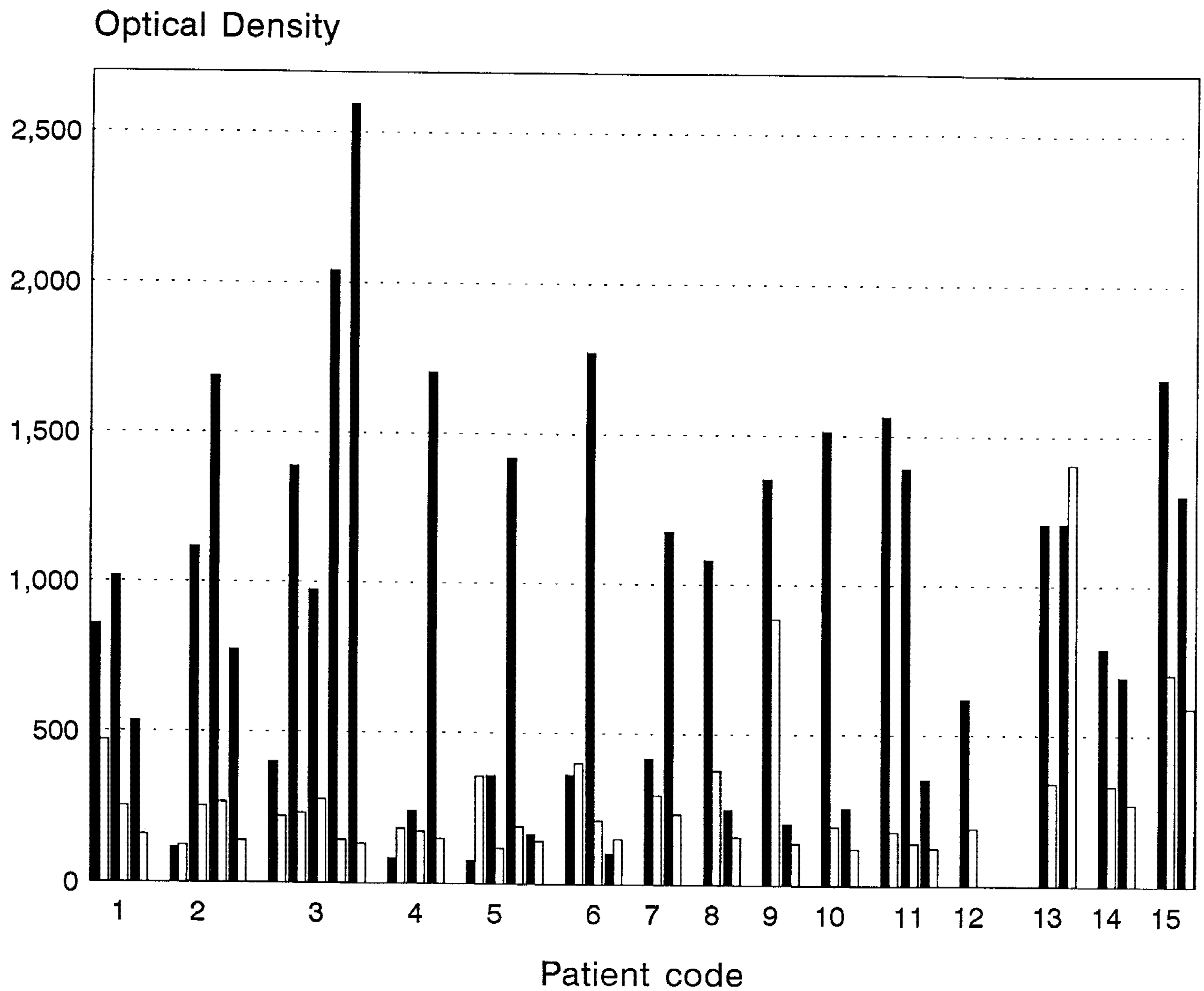

Fig. 2. HBeAg quantitation in samples from early acute hepatitis B by the standard assay (closed bars) and selection assay (open bars) in patients with acute self limiting disease (1–12) and those that developed chronicity (13–15). The results of the selection assay were normalized for HBeAg concentration in the standard assay. Samples from the chronic cases correspond to weeks 28 and 32 (patient 13), weeks 14 and 20 (patient 14), and weeks 15 and 17 (patient 15) from Figure 1. Samples from patients 1–12 were taken with 1–3-week intervals.

TABLE II. Percentage Inhibition of HBeAg Signal (Selection Assay) in Samples From the Acute Phase of HBV Infection in Two Patients Who Developed Chronicity

HBeAg$^+$ sample	Inhibition with HBsAg$^+$/HBeAg$^+$	HBsAg$^+$/HBeAg$^-$	HBsAg$^-$/anti-HBe$^+$	Sample
Patient 13	44%			Patient 15[a,b]
	100%	100%	100%	Patient 11
Patient 15	82%			Patient 15[a]
	79%	72%	79%	Patient 2

[a]Serum samples used for inhibition were HBeAg-negative in the selection assay.
[b]Sample taken 3 years after start of infection.

hepatitis B occupied with anti-HBe, which is not detected by standard anti-HBe assays. The enhanced signal in our selection assay could be an indication of limited immune activity.

Variable results of HBeAg assays were also described by Maruyama et al. [1992, 1993, 1994], who developed an assay that detected HBeAg immune complexes (IC) in the presence of excess of circulating HBeAg. The prevalence of HBeAg IC was low in acute hepatitis but widespread in chronic hepatitis B [Maruyama et al., 1994]. This is not confirmed by our observations.

Since the epitope related to monoclonal HBOT.95a differs from that of Maruyama et al. [1994], these immune complexes may differ in composition compared to those in the present study. Further exploration of the

early phases of acute hepatitis B infection, including the response on HBeAg epitopes, may help to find patients with an increased risk for chronicity, with the aim of starting antiviral therapy before viral replication rebound [Vinogradova et al., 1996].

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